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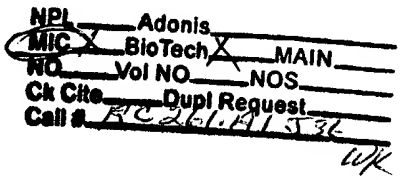

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Jpn J Cancer Res. 1996 Jul;87(7):757-64.

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Exp Hematol. 1996 Feb;24(2):360-3.

Eur J Immunol. 1993 Jan;23(1):186-90.

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J Natl Cancer Inst. 1995 Jun 7;87(11):809-16.

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Jpn. J. Cancer Res. 87, 757–764, July 1996

Transduction of the Macrophage Colony-stimulating Factor Gene into Human Multidrug Resistant Cancer Cells: Enhanced Therapeutic Efficacy of Monoclonal Anti-P-glycoprotein Antibody in Nude Mice

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To develop a therapeutic modality for overcoming multidrug-resistant (MDR) cancer with anti-MDR1 antibody, we examined the effect of macrophage colony-stimulating factor (M-CSF) gene transfection into MDR AD10 cells on therapy of MDR cancer with anti-MDR1 antibody (MRK17) in nude mice. MDR human ovarian cancer (AD10) cells were transduced with the human M-CSF gene inserted into an expression vector to establish gene-modified cells capable of producing low (ML-AD10), intermediate (MM-AD10) and high (MH-AD10) amounts of M-CSF. Systemic administration of MRK17 resulted in significant dose-dependent inhibition of subcutaneous growth of ML-AD10 tumors. In contrast, systemic administration of recombinant M-CSF in combination with MRK17 did not augment the therapeutic efficacy of MRK17 alone, but rather promoted the growth of the parent AD10 cells. To test the efficacy of *in vivo* M-CSF gene therapy combined with antibody, we mixed the parent AD10 cells with MH-AD10 cells producing a large amount of M-CSF, and inoculated the mixed cells subcutaneously. Treatment with MRK17 inhibited growth of the mixed cells more than that of the parent cells alone. Thus, combined therapy with anti-MDR1 mAb and M-CSF gene modification of MDR cancer cells may provide a new immunotherapeutic modality for overcoming MDR in humans.

Key words: Anti-P-glycoprotein antibody — Multidrug resistance — M-CSF — Gene therapy

Multidrug resistance (MDR) of tumors is a major obstacle to successful cancer chemotherapy. MDR1 (P-glycoprotein), one of the key molecules in MDR, has been shown to bind anti-cancer drugs,[1-3] and to function as an ATP-driven efflux pump for various cytotoxic drugs.[4,5] The expression of MDR1 was found to be elevated in intrinsically drug-resistant cancers as well as in some tumors that acquired drug resistance during chemotherapy.[6,7] Recently, even low levels of MDR1 expression were shown to serve as a marker of resistance to combination chemotherapy in human ovarian cancer and small cell lung cancer.[8] Thus, from an immunotherapeutic point of view, P-glycoprotein seems to be a good molecular target for the selective killing of tumor cells expressing it. There is encouraging evidence that murine monoclonal antibodies (mAbs) (MRK16, MRK17) raised specifically against human MDR1 induce lysis of MDR cancer cells *in vitro* by human effector cells,[9,10] and cause rapid regression of established s.c. MDR tumors, regression being complete in some animals.[11]

Macrophage colony-stimulating factor (M-CSF) is known to augment antibody-dependent monocyte-mediated cytotoxicity (ADCC) against cancer cells by increasing the number and affinity of Fc receptors expressed on the monocytes.[12-14] We recently reported that M-CSF-primed blood monocytes show enhanced ADCC in the presence of anti-MDR1 mAb against MDR human cancer cells expressing MDR1.[10] These findings indicated that one potentially important mechanism for the *in vivo* anti-tumor effect of M-CSF is its ability to augment monocyte-mediated anti-MDR1 mAb-dependent cytotoxicity against MDR cancer cells. To augment the *in situ* ADCC activity mediated by monocytes and macrophages, we inserted the M-CSF gene into human MDR AD10 cancer cells to establish M-CSF gene-modified cell lines with differing abilities to produce M-CSF, and found that tumor-derived M-CSF could augment anti-MDR1 mAb-dependent monocyte-mediated killing of MDR cancer cells.[15] Interestingly, we also found an inverse relationship between the ability of the cells to produce M-CSF and their tumorigenicity.[15] In this paper, we report the combined effects of M-CSF gene-transduction into MDR cancer cells and systemic administration of anti-MDR1 mAb in the therapy of human MDR cancer in nude mice.

[4] To whom request for reprints should be addressed.

MATERIALS AND METHODS

Cell lines The human ovarian tumor A2780 and its adriamycin-resistant variant, 2780AD (AD10) cells, were kindly supplied by Drs. R. F. Ozols and T. C. Hamilton, National Cancer Institute. The characteristics of these cell lines have been reported.[16]

M-CSF gene-modified AD10 cells capable of producing various amounts of M-CSF were cloned as described previously.[15] Briefly, the pRc/CMV-MCSF plasmid was constructed by cloning a 1.8-kb *Eco*RI cDNA fragment containing the complete coding region of human M-CSF[17] into the *Hin*dIII cloning site of the pRc/CMV vector (Invitrogen, San Diego, CA). Transduction was performed by the calcium phosphate precipitation technique.[18] Selection of transfectants in RPMI 1640 with 10% FBS (fetal bovine serum) containing 500 μg/ml of G418 was started two days after transduction. G418-resistant colonies were pooled to yield a bulk transduced culture, which was then cloned by limiting dilution to yield several lines. All cell cultures were then tested for secretion of M-CSF. Tumor cell clones producing low (ML-AD10), intermediate (MM-AD10) and high (MH-AD10) levels of M-CSF and the parent AD10 cells were used in further experiments. For quantitative measurement of M-CSF production.[15] these cells (103 cells/ml) were incubated for 7 days and the supernatants were harvested. The amounts of M-CSF produced by the parent AD10, ML-AD10, MM-AD10 and MH-AD10 cells were <0.2, 1.6, 8.5 and 100 ng/ml, respectively.

Reagents FBS and G418 were purchased from Gibco (Grand Island, NY). A recombinant form of human M-CSF (specific activity, 0.8×10^6 U/mg protein) was obtained from Otsuka Pharmaceutical Co. (Tokushima). Anti-MDR1 mAb MRK17 (IgG1) was prepared as described previously.[3,11] None of these materials contained endotoxins, as judged by amebocyte assay (Seikagaku Kogyo, Tokyo; minimal detection level 0.3ng/ml).

Isolation and culture of mouse peritoneal macrophages Peritoneal macrophages were harvested by lavage of the peritoneal cavity of nude mice with prewarmed PBS, and were washed twice with phosphate-buffered saline, and resuspended in CRPMI1640. The cells were then incubated in suspension in medium with or without human recombinant M-CSF for three days, resuspended in CRPMI1640 at various concentrations, and plated in 96-well Microtest III plates (Falcon, Oxford, CA). After one hour, nonadherent cells were removed by washing with the medium. At this point the purity of the macrophages was >99% as judged from their morphology and the result of nonspecific esterase staining. These macrophage-rich cultures were used as effector cells.

Antibody-dependent cell-mediated cytotoxicity (ADCC) The target cells were labeled with ^{51}Cr as described before.[9,10] Various numbers of effector cells (macrophage monolayers) in 96-well Microtest III plates were mixed with suspensions (100 μl) of 1×10^4 ^{51}Cr-labeled target cells that had been incubated at 37°C for 30 min with various concentrations of anti-MDR1 antibody (MRK17). The plates were centrifuged for 3 min at 100 $\times g$, and then incubated for 4 h. The radioactivities of 100 μl samples of supernatant obtained by centrifugation were then counted in a gamma counter. Determinations were carried out in triplicate. The percentage of specific cytolysis was calculated from the release of ^{51}Cr from test samples and control samples as follows:

$$\% \text{ Specific release} = (E - S) / (M - S) \times 100$$

where E is the release in the test sample (cpm in the supernatant from target cells incubated with effector cells and test antibody), S is the spontaneous release (cpm in the supernatant from target cells incubated with medium alone), and M is the maximum release (cpm released from target cells lysed with 1 *N* HCl). The spontaneous release observed with different target cells ranged from 5% to 17% (total lysis).

Determination of M-CSF M-CSF was measured by radioimmunoassay (sensitivity limit, 0.1 ng/ml) as described before.[19] Briefly, duplicate samples (100 μl) were mixed with ^{125}I-M-CSF (1000 cpm/100 μl) and then with a 2000-fold dilution of anti-M-CSF antiserum (200 μl). The anti-M-CSF antibody binding reaction attained equilibrium within 20 h at room temperature or within 48 h at 4°C. After incubation, bound antibody was separated from free ^{125}I-M-CSF by addition of 100 ml of normal rabbit serum diluted 400 times with PBS, 100 μl of anti-rabbit IgG serum diluted 40 times with PBS, and 1.0 ml of 6 % polyethylene glycol (MW 8000) in PBS. The tubes were shaken and centrifuged at 1000*g* for 15 min at 4°C. The supernatants were removed by aspiration, and the radioactivities of the precipitates were counted for 1 min in an automated gamma spectrometer.

Animal studies Female BALB/c-nu mice and AF-nu mice (3 and 8 weeks old) were purchased from Charles River Breeding Laboratories (Tokyo) and maintained at the Cancer Institute under standard conditions according to the Institutional Guidelines. The tumorigenic activities of control and M-CSF gene-transfected cells were assayed by injecting 0.1 ml of cell suspension s.c. into the right flank of 3-week-old mice (5–7 mice/group) via a 26-gauge needle on a 1-ml syringe. The parent AD10 cells were previously found to grow progressively in 69% of 3-week-old mice.[15] In preliminary experiments, we observed that when 2×10^7 parent AD10 cells were injected s.c. into 8-week-old nude mice, all the mice developed palpable tumors. In subsequent experiments, 8-week-old mice were used unless otherwise described. For therapy of s.c. tumors, 2×10^7 AD10 cells mixed with or without 2.4×10^5 or 7.2×10^5 MH-AD10 cells were inoculated into the right flank, and i.v. injections of MKR17 were

given on days 2 and 7 or days 9 and 14 after tumor inoculation. To test the effect of systemic administration of rM-CSF on the growth of AD10 cells, we injected 1 or 10 μg/ml of rM-CSF i.v. on days 8, 9, 10, 13, 14 and 15 with 10 μg/ml of MRK17 on days 9 and 14. Tumor growth was examined by palpation every day and the tumor volume was measured every 3–4 days for the indicated period. The experiment was carried out at least twice, producing similar results.

Statistical analysis The statistical significance of differences between groups was analyzed by the use of Student's *t* test (two-tailed).

RESULTS

Anti-MDR1 mAb-dependent killing of human MDR AD10 cells by murine peritoneal macrophages First, we examined whether M-CSF gene transduction of human MDR AD10 cells could affect their susceptibility to ADCC mediated by murine macrophages. The results are shown in Table I. Murine peritoneal macrophages were significantly cytotoxic to parent and M-CSF gene-modified AD10 cells at all E/T ratios tested in the presence of 0.1 μg/ml of MRK17 ($P<0.05$). There were no significant differences among the clones in their susceptibility to ADCC by murine macrophages.

Next, we tested whether human M-CSF could enhance anti-MDR1 mAb-dependent cytotoxicity mediated by murine macrophages. Peritoneal macrophages were incubated in the presence of 5000 U/ml of human M-CSF for 3 days and assayed for MRK17-dependent cellular cytotoxicity. Table II shows that treatment with human M-CSF of mouse peritoneal macrophages resulted in higher ADCC activity than that of medium-treated macrophages against both parent and M-CSF gene-transfected AD10 cells.

Effect of combined systemic injections of recombinant M-CSF and MRK17 on growth of AD10 cells First, we examined the effect of the time of treatment with MRK17 on palpable tumor formation by AD10 cells.

Table I. Anti-MDR1 mAb-dependent Killing of Human MDR AD10 Cells by Mouse Peritoneal Macrophages

E/T ratio	MRK17 (0.1 μg/ml)	% Specific cytotoxicity against[a]		
		Parent AD10	ML-AD10	MH-AD10
10	−	3.7±0.7[b]	2.8±0.9	3.7±1.8
	+	9.7±0.9[c]	7.3±2.3[c]	7.6±0.3[c]
20	−	9.2±0.4	8.7±1.1	5.8±1.0
	+	19.9±3.0[c]	19.9±2.3[c]	15.7±1.8[c]
40	−	16.6±2.6	18.5±1.5	11.3±1.6
	+	52.4±0.8[c]	47.5±3.8[c]	53.3±1.6[c]

a) Peritoneal macrophages of nude mice were assayed for anti-MDR1 mAb (MRK17)-dependent cytotoxicity against parent and M-CSF gene-modified MDR cancer cells at the indicated E/T ratios.
b) Mean±SD for triplicate cultures.
c) Significantly different from that for macrophages without MRK17 at the corresponding E/T ratio ($P<0.05$).

Table II. Anti-MDR1 mAb-dependent Killing of Human MDR AD10 Cells by Mouse Peritoneal Macrophages and Its Augmentation by M-CSF

MRK17 (0.1 μg/ml)	Treatment of macrophages[a]	% Specific cytotoxicity against		
		Parent AD10	ML-AD10	MH-AD10
−	Medium	2.6±3.0[b]	1.3±0.2	4.8±4.0
−	M-CSF	3.1±2.2	0.1±0.6	3.5±1.0
+	Medium	22.7±0.8	23.1±1.0	24.6±1.2
+	M-CSF	33.1±2.4[c]	31.3±1.6[c]	29.8±2.1[c]

a) Peritoneal macrophages of nude mice were incubated for 3 days in medium with or without the indicated concentrations of human M-CSF (5000 U/ml) before assay of anti-MDR1 mAb (MRK17)-dependent cytotoxicity against parent and M-CSF gene-modified MDR cancer cells at an E/T ratio of 20:1.
b) Mean±SD for triplicate cultures.
c) Significantly different from that for macrophages without M-CSF treatment in the presence of MRK17 ($P<0.05$).

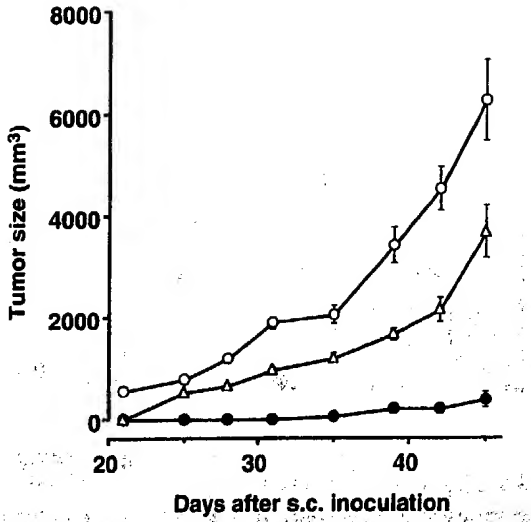

Fig. 1. Effect of time of treatment with MRK17 on s.c. growth of human MDR cancer (AD10) cells. All mice (6 or 7 mice/group) were inoculated s.c. with 2×10^7 AD10 cells on day 0 and then given i.v. injections of PBS (○) or 10 μg/ml of MRK17 (●) on days 2 and 7. One group of mice was also injected with 10 μg/ml of MRK17 on days 9 and 14 to test its growth inhibitory effect at a late phase (△). Bars represent mean±SE.

After s.c. inoculation of AD10 cells (2×10^7 cells), PBS with or without MRK17 (10 μg) was injected intravenously into 8-week-old mice on days 2 and 7, and also in some cases on days 9 and 14. The results are shown in Fig. 1. The parent AD10 cells formed palpable tumors in all mice and grew progressively. Under the same experimental conditions, treatment with MRK17 on days 2 and 7 resulted in almost complete inhibition of palpable tumor formation with 60% tumor uptake. When MRK17 was injected intravenously on days 9 and 14, moderate inhibition of tumor growth was seen in all the mice with palpable tumors.

Second, we examined whether combined treatment with MRK17 and rM-CSF instead of M-CSF gene transduction could be more effective therapeutically than either MRK17 or recombinant M-CSF alone for inhibition of tumor formation of AD10 cells inoculated s.c. into nude mice. For this, AD10 cells (2×10^7) were injected s.c. into nude mice, and then the mice were given i.v. injections of M-CSF on days 8, 9, 10, 13, 14 and 15 with or without MRK17 on days 9 and 14 after tumor inoculation. Tumor growth was examined by palpation for 40 days. Treatment with MRK17 alone resulted in 75% tumor formation by AD10 cells (Table III). Addition of recombinant M-CSF to MRK17 did not cause inhibition of tumor formation, but rather increased

Table III. Effect of Combined Systemic Injections of M-CSF and Anti-MDR1 mAb (MRK17) on Growth of Human MDR AD10 Cell in Nude Mice

Treatment	Mice with tumor/total		Total (%)
	Exp. 1	Exp. 2	
PBS	6/6	6/6	12/12 (100)
M-CSF 1 μg	5/5	5/6	10/11 (91)
M-CSF 10 μg	6/6	6/6	12/12 (100)
MRK17 10 μg	5/6	4/6	9/12 (75)
M-CSF + MRK17 1 μg 10 μg	5/6	5/6	10/12 (83)
M-CSF + MRK17 10 μg 10 μg	6/6	5/6	11/12 (92)

All mice were injected s.c. in the right flank with 2×10^7 AD10 cells on day 0. On days 9 and 14 after tumor inoculation, groups of mice were treated i.v. with PBS or 10 μg of MRK17 and also treated with 1 or 10 μg of M-CSF on days 8, 9, 10, 13, 14 and 15. Tumor uptake was determined on day 56.

tumor uptake over that on treatment with MRK17 alone (Table III). When the sizes of growing tumors were also measured for 40 days, systemic treatment with MRK17 alone resulted in significant suppression of tumor formation by AD10 cells in nude mice, but that with recombinant M-CSF (10 μg) alone did not result in significant suppression ($P<0.05$). Moreover, recombinant M-CSF did not increase, but rather reduced the anti-tumor effect on growth of AD10 cells by MRK17 (Fig. 2).

Therapy of low-M-CSF-producing AD10 (ML-AD10) cells with anti-MDR1 mAb (MRK17) Our previous findings[15] showed that when human MDR cancer cells (2×10^7 cells/mouse) with or without M-CSF gene-modification were injected s.c. into 3-week-old nude mice, injection of parent AD10 cells or those transfected with a control gene resulted in palpable tumor outgrowth in 69% of the mice tested. ML-AD10 cells producing a small amount of M-CSF also formed palpable tumors in all recipient mice. MM-AD10 cells and MH-AD10 cells formed tumors in only 5 of 13 mice (38%), and 2 of 13 mice (15%), respectively. Under the same experimental conditions, we examined whether systemic administration of anti-MDR1 mAb (MRK17) could inhibit the s.c. growth of ML-AD10 cells producing a low amount of M-CSF in nude mice. For this, 2×10^7 cells were inoculated s.c. into mice, and on days 2 and 7, these mice rceeived i.v. injections of MRK17 at concentrations of 3 to 100 μg/body. The tumor diameters were measured serially and the results are shown in Fig. 3. Intravenous administrations of MRK17 at doses of more than 3 μg resulted in significant inhibition of the growth of palpable tumors in a dose-dependent manner ($P<0.05$). Maximal inhibition of the subcutaneous growth of ML-AD10 cells

was seen in mice that received injections of 30 μg of MRK17 mAb.

Therapy by MRK17 of human MDR cancer consisting of a mixture of parent AD10 cells and MH-AD10 cells We examined whether gene-modification of a few cells within the growing MDR cancer was sufficient in treatment with MRK17 to inhibit growth of MDR cancer in nude mice. For this, we mixed the parent AD10 cells with MH-AD10 cells producing a large amount of M-CSF, inoculated the mixture s.c. into 8-week-old female nude

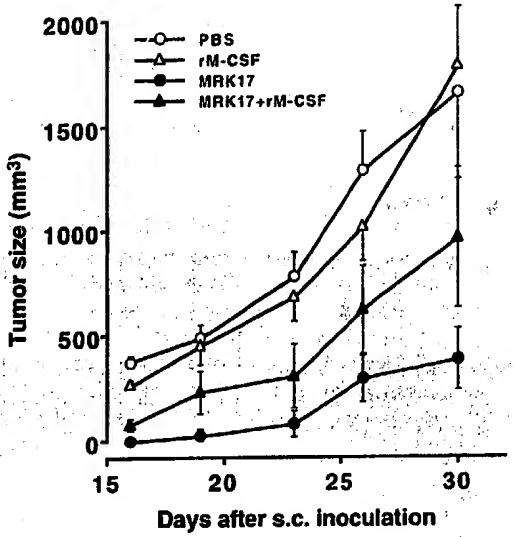

Fig. 2. Effect of combined systemic injections of recombinant human M-CSF and anti-MDR1 mAb (MRK17) on growth of human MDR AD10 cells in nude mice. All mice were injected s.c. in the right flank with 2×10^7 AD10 cells on day 0. On days 9 and 14 after tumor inoculation, groups of mice were treated i.v. with PBS or 10 μg of MRK17 and also treated with 10 μg of recombinant M-CSF (rM-CSF) on days 8, 9, 10, 13, 14 and 15. Bars represent mean ± SE.

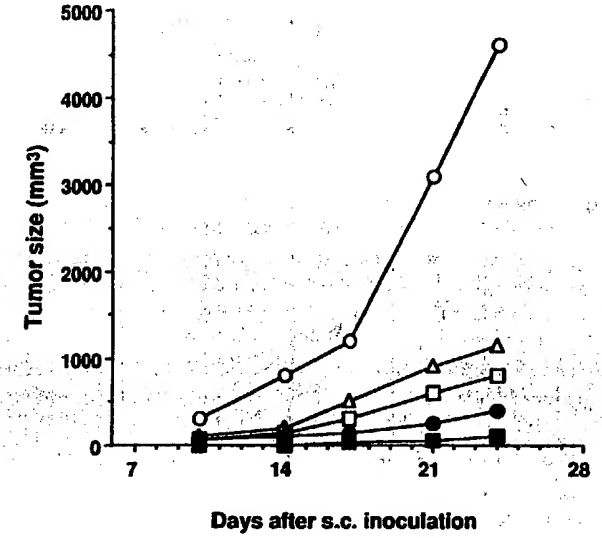

Fig. 3. Therapy of low-M-CSF-producing AD10 (ML-AD10) cells with anti-MDR1 mAb (MRK17). ML-AD10 cells (2×10^7 cells) were inoculated s.c. into groups of mice (6–7 mice/group), and on days 2 and 7, the mice were given i.v. injections of PBS (○) or MRK17 at a concentration of 3 (△), 10 (□), 30 (■) or 100 (●) μg/mouse. Tumor diameters were measured serially until day 24.

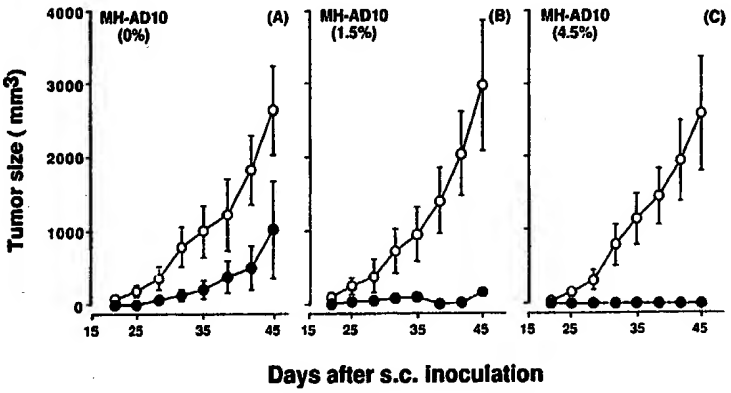

Fig. 4. Therapy with MRK17 of human MDR cancer consisting of a mixture of the parent AD10 cells and MH-AD10 cells. Mixtures of the parent AD10 cells (2×10^7) and the indicated percentages of AD10 cells producing a large amount of M-CSF (MH-AD10 cells) were inoculated s.c. into groups of mice (6 mice/group), and then PBS (○) or MRK17 (10 μg/body) (●) was injected on days 2 and 7. Tumor volumes were measured every 3–4 days until day 56. Bars represent mean ± SE.

mice, and injected MRK17 (10 μg) on days 2 and 7. Results are shown in Fig. 4. When MRK17 was not administered, AD10 cells mixed with or without MH-AD10 cells grew progressively in all the mice. Systemic treatment with MRK17 (10 μg) significantly inhibited the growth of AD 10 cells alone after 28 days post tumor inoculation ($P<0.05$). Under the same experimental conditions, tumor formation of AD10 cells mixed with 1.5% or 4.5% of MH-AD10 cells was almost completely inhibited by MRK17 treatment as compared to that without mAb treatment.

DISCUSSION

In the present study, we demonstrated that M-CSF gene transduction into MDR AD10 cells, but not systemic injections of M-CSF, enhanced the therapeutic efficacy of anti-MDR1 mAb (MRK17) for human MDR cancer.

There is accumulating evidence that the use of antibodies may be effective in cancer treatment.[20] mAbs (MRK16 and MRK17) directed against P-gp expressed on MDR cancer cells are expected to be useful in the immunotherapy of human MDR malignant cells,[11] but possible side effects of anti-MDR1 antibodies administered to humans should be carefully studied because of MDR1 expression in normal tissues. We previously demonstrated that mAbs (MRK16 and MRK17) were effective in inducing human monocyte-mediated killing of MDR1-positive cancer cells and that M-CSF enhanced monocyte-mediated ADCC against human MDR cancer cells through enhancement of CD16, CD32 and CD64 expressions on monocytes,[10] suggesting an important anti-tumor role of M-CSF. The present study was designed to determine the therapeutic efficacy of M-CSF gene transduction into human MDR cancer cells in combination with anti-MDR1 mAb. In this study we found that human MDR ovarian cancer (AD10) cells growing subcutaneously in nude mice were susceptible to the systemic administration of anti-MDR1 mAb, and that MRK17 in combination with transduction of the M-CSF gene into MDR cancer cells was more therapeutically effective than either MRK17 or M-CSF gene transduction alone.

Macrophages are known to have dual roles in *in vivo* tumor growth and progression.[21-23] M-CSF was found to induce tumoricidal activity of monocytes.[13,24] Moreover, administration of high doses of recombinant M-CSF was also shown to be effective in inducing tumor regression in experimental murine metastasis models.[25] We found that MH-AD10 cells capable of producing a large amount of M-CSF had greatly reduced tumorigenicity when the cells were inoculated s.c. in nude mice.[15] In contrast, gene-modified ML-AD10 cells capable of producing a small amount of M-CSF were found to form tumors in all mice examined, whereas the parent cells grew in only 69% of the recipients to yield palpable tumors.[15] The presence of a small amount of M-CSF at the tumor growth site might attract and influence the functions of tumor-associated macrophages which provide optimal micro-environment conditions for growth of AD10 tumors. Under these experimental conditions, treatment with MKR17 caused significant, dose-dependent eradication of ML-AD10 tumors (Fig. 3). This finding suggests that even if low levels of M-CSF are produced locally at the tumor site to promote tumor growth, combined use of anti-MDR1 antibody might be therapeutically useful for *in situ* destruction of MDR cancer cells.

We found that systemic administration of large amounts of recombinant human M-CSF was not effective in inhibiting the tumor formation of MDR ovarian cancer AD10 cells in nude mice, and that systemic administration of recombinant M-CSF in combination with anti-MDR1 mAb did not increase the anti-tumor effect on growth of AD10 cells inoculated s.c. in nude mice over that of anti-MDR1 mAb alone (Table III, Fig. 2). The reason for this failure is unknown at present. Nevertheless, there are several possible explanations. Firstly, systemic administration of exogeneous M-CSF might stimulate ovarian cancer growth *in vivo*, because a potential effect of exogeneous M-CSF on ovarian cancer cells has been reported in an *in vitro* model of the malignant phenotype of invasion.[25] Indeed, high levels of M-CSF in serum seem to imply a poor prognosis in patients with ovarian cancer.[26] Secondly, systemic administration of recombinant M-CSF might cause monocytosis in the circulation, but not allow monocytes to extravasate to the tumor site. Another possible explanation is that the lack of inhibitory effect of systemic M-CSF administration on the growth of s.c.- inoculated tumor cells might be due to insufficient M-CSF to stimulate macrophages at the local tumor site. This was not the case, however, in a metastatic model of melanoma because systemic administration of M-CSF reduced pulmonary or liver metastases.[27,28] These findings suggest that systemic administration of M-CSF might reduce the metastatic spread of cancer cells because systemically activated monocyte-macrophages come in contact with cancer cells passing through the blood stream. MKR17 was therapeutically effective when given on days 2 and 7 after AD10 inoculation (Fig. 4), but it had little effect when given on days 9 and 14 (Fig. 2). Further study is required to find optimal conditions for combined treatment with recombinant M-CSF and anti-MDR1 antibody in terms of doses and timing of each agent.

Recently much attention has been paid to the development of methods for gene therapy of cancer.[29-32] In murine systems, there is encouraging evidence that vari-

ous methods of delivery of biologically active genes into animal cells are highly effective.[33-35] A successful gene delivery system may lead to gene therapeutic strategies for introduction of the M-CSF gene into MDR cancer cells which could be followed by treatment with anti-MDR1 mAb. With regard to gene targeting to cancer cells *in vivo*, even if an appropriate delivery system can be successfully designed, gene targeting does not seem to be 100% efficient in modifying the tumor cells. Presumably, some cancer cells can be gene-modified by a delivery system using physical or biological vectors. So, it is very important to know whether gene-modification of a few cells within a growing MDR cancer is sufficient for inhibition of growth of the MDR cancer in nude mice. To test this, we mixed the parent AD10 cells with MH-AD10 cells producing a large amount of M-CSF to provide the same level of M-CSF production as that by low-producing (ML-AD10) cells. For this, the mixed cells contained 1.5% and 4.5% MH-AD10 cells. All the recipient nude mice had palpable tumors even after s.c. inoculation of the parent AD10 cells with or without MH-AD10 cells. Treatment with MRK17 mAb was markedly more effective in inhibiting tumor formation of AD10 cells mixed even with 1.5% MH-AD10 cells than that of AD10 cells alone (Fig. 4). These findings strongly indicate that if a few cells within MDR tumors can be gene-targeted in a particular way to produce M-CSF, anti-MDR1 mAb (MRK17) should be therapeutically more useful for eradicating human MDR cancer.

In summary, local production of M-CSF at the site of tumor growth is a prerequisite for successful eradication of MDR ovarian cancer by anti-MDR1 mAb. The present findings suggest that combined treatment with M-CSF gene transduction and anti-MDR1 mAb should be effective against MDR cancer in humans.

ACKNOWLEDGMENTS

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Direct stimulation of cells expressing receptors for macrophage colony-stimulating factor (CSF-1) by a plasma membrane-bound precursor of human CSF-1.

Stein J, Borzillo GV, Rettenmier CW.

Department of Hematology/Oncology, St Jude Children's Research Hospital, Memphis, TN.

Secreted forms of macrophage colony-stimulating factor (M-CSF or CSF-1) are generated by proteolytic cleavage of membrane-bound glycoprotein precursors. Alternatively spliced transcripts of the human CSF-1 gene encode at least two different transmembrane precursors that are differentially processed in mammalian expression systems. The larger precursor rapidly undergoes proteolysis to yield the secreted growth factor and does not give rise to forms of CSF-1 detected on the cell surface. By contrast, the smaller human CSF-1 precursor is stably expressed on the plasma membrane where it is inefficiently cleaved to release a soluble molecule. To determine whether the smaller precursor is biologically active on the cell surface, mouse NIH-3T3 fibroblasts expressing the different forms of human CSF-1 were killed by chemical fixation and tested for their ability to support the proliferation of cells that require this growth factor. Only fixed cells expressing human CSF-1 precursors on their surface stimulated the growth in vitro of a murine macrophage cell line or normal mouse bone marrow-derived mononuclear phagocytes. The ability of these nonviable fibroblasts to induce the proliferation of CSF-1-dependent cells was not mediated by release of soluble growth factor, required direct contact with the target cells, and was blocked by neutralizing antiserum to CSF-1. These results demonstrate that the cell surface form of the human CSF-1 precursor is biologically active and indicate that plasma membrane-bound growth factors can functionally interact with receptor-bearing targets by direct cell-cell contact.

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Human macrophage colony-stimulating factor is expressed at and shed from the cell surface.

Tuck DP, Cerretti DP, Hand A, Guha A, Sorba S, Dainiak N.

Department of Medicine, Royal Victoria Hospital, McGill University, Montreal, Canada.

Surface membrane-associated growth factors are being recognized as important for developmental processes, including cell assembly, differentiation, and growth. To investigate the role of membrane-bound macrophage colony-stimulating factor (M-CSF) in myelopoiesis, and whether this factor is released from the cell surface in association with shed membrane-derived vesicles, COS-1 cells were transfected with cDNAs for M-CSF-tau (containing the transmembrane domain) or a soluble mutant form of the molecule lacking the transmembrane domain ([s]M-CSF-alpha). COS-1 cells transfected with either cDNA released activity into the spent culture medium. Conditioned medium was separated by centrifugation into supernatants and pellets were found to contain plasma membrane-derived vesicles by transmission electron microscopy. When medium fractions were assayed in marrow cultures, activity was localized to shed plasma membrane-derived vesicles in medium conditioned by cells transfected with cDNA for M-CSF-tau and in the vesicle-free supernatants of medium conditioned by cells transfected with cDNA for [s]M-CSF-alpha. In addition, nuclear, mitochondrial, and plasma membrane subfractions of stably transfected cells were prepared and assayed for activity. Concentration-dependent stimulation of macrophage colony formation was observed with purified plasma membranes (but not nuclear or cytosolic proteins) from cells transfected with cDNA for M-CSF-tau. By contrast, membranes from untransfected cells and cells transfected with cDNA for [s] M-CSF-alpha or control DNA expressed no activity. Together, the data indicate that human M-CSF is expressed at the cell surface and exfoliated in association with surface membrane-derived vesicles.

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Characterization of tumor-necrosis-factor-gene-transduced tumor-infiltrating lymphocytes from ascitic fluid of cancer patients: analysis of cytolytic activity, growth rate, adhesion molecule expression and cytokine production.

Itoh Y, Koshita Y, Takahashi M, Watanabe N, Kohgo Y, Niitsu Y.

Fourth Department of Internal Medicine, Sapporo Medical University School of Medicine, Japan.

We characterized tumor-infiltrating lymphocytes (TIL) from ascites of patients with ovarian or pancreatic cancer in which the human tumor necrosis factor (TNF) gene was successfully transduced with retrovirus vector. The TNF-gene-transduced TIL (TNF-TIL) from these patients showed a higher level of TNF production and higher cytotoxic activity against K562 and Daudi cells than did neomycin-phosphotransferase-gene-transduced TIL (neo-TIL). Of these TIL preparations, only that from pancreatic cancer was further characterized since it was collected in a relatively large amount. In spite of the fact that the autologous tumor cells showed resistance to soluble TNF, the TNF-TIL clearly demonstrated enhanced cytotoxicity against them as compared with neo-TIL. The enhanced cytotoxicity was ascribed to autocrine effects of secreted TNF on TIL, which included augmentation of adhesion molecule (CD2 and CD11a) and interleukin-2 receptor expression, and elevation of production of interferon gamma, lymphotoxin and granulocyte/macrophage-colony-stimulating factor and its paracrine effect on target cells to facilitate them to be more susceptible to TIL.

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Expression of surface lymphotoxin and tumor necrosis factor on activated T, B, and natural killer cells.

Ware CF, Crowe PD, Grayson MH, Androlewicz MJ, Browning JL.

Division of Biomedical Sciences, University of California, Riverside 92521-0121.

The expression of membrane-associated forms of lymphotoxin (LT) and TNF were examined on cell lines of T, B, and myeloid origin, IL-2 dependent T cell clones, and peripheral blood lymphocytes. Inducible and constitutive patterns of surface LT expression were found on T cells as exemplified by the II-23.D7, a CD4+T cell hybridoma, and HUT-78, a T cell lymphoma. Phorbol ester induced surface LT expression on Ramos, an EBV transformed B cell line, but at a slower rate of appearance when compared to the II-23.D7. Secretion of LT was rapidly inducible by phorbol ester in II-23.D7 and also in HUT-78 but with slower kinetics; surface LT expression continued in both lines after secretion had ceased. Low levels of membrane TNF were transiently induced on II-23.D7 and HUT-78, but none was observed on Ramos. Peripheral blood monocytes and some myeloid tumor lines did not express surface LT. Several T cell clones expressed surface LT after Ag-specific stimulation, and expression persisted several days. Stimulation through the TCR or by IL-2 rapidly induced surface LT on resting peripheral T cells and CD56+ NK cells; pokeweed mitogen activation induced expression on CD20+ B cells. Consistent with previous results, immunoprecipitation with anti-LT mAb showed that LT was complexed with a distinct 33 kDa glycoprotein (p33) on cells that expressed surface LT, whereas secreted LT was not associated with p33. Surface and secreted modes of LT expression by activated T, B, and NK cells suggests that LT can be utilized as either a localized or diffusible mediator in immune responses.

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Osteoclast differentiation factor (ODF) induces osteoclast-like cell formation in human peripheral blood mononuclear cell cultures.
Biochem Biophys Res Commun. 1998 May 8;246(1):199-204.
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A comparative review of colony-stimulating factors.
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Proc Natl Acad Sci U S A. 1997 May 13;94(10):5296-301.
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Detection of a membrane-associated cytokine (macrophage colony stimulating factor) using a modified commercially available ELISA.
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Differential effects of macrophage- and granulocyte-macrophage colony-stimulating factors on cytokine gene expression during rat alveolar macrophage differentiation into multinucleated giant cells (MGC): role for IL-6 in type 2 MGC formation.
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Human macrophage colony stimulating factor (M-CSF): alternate RNA splicing generates three different proteins that are expressed on the cell surface and secreted.

Cosman D, Wignall J, Anderson D, Tushinski J, Gallis B, Urdal D, Cerretti DP.

Department of Molecular Biology, Immunex Corporation, Seattle, Washington 98101.

Human macrophage colony stimulating factor (M-CSF) cDNA clones were isolated from a pancreatic carcinoma cell line. Three different classes of M-CSF precursor protein (256, 554 and 438 amino acids in length) were predicted to be encoded by these cDNAs. Two of these, that we designate M-CSF alpha and M-CSF beta have already been described. The third, M-CSF gamma represents a novel class of M-CSF cDNA. All three precursors share a 32 amino acid signal sequence and the first 149 amino acids of the mature protein. At this position, M-CSF beta and gamma have insertions of 298 and 182 amino acids relative to M-CSF alpha. The first 182 amino acids of these insertions are shared between M-CSF beta and gamma. All three precursors share the C-terminal 75 amino acids that encode the transmembrane and cytoplasmic domains. Expression of all three cDNAs in COS-7 monkey kidney cells gave rise to soluble M-CSF activity, associated with proteins of subunit molecular weight 44 Kda (beta and gamma) or 28 Kda (alpha). In addition, M-CSF proteins could be detected on the surface of the transfected cells by indirect immunofluorescence. When the transmembrane and cytoplasmic domains of M-CSF alpha were removed by introducing a stop codon after amino acid 190, no membrane-bound M-CSF could be detected, but the truncated protein was secreted efficiently and was biologically active. This suggests that all three forms of M-CSF can exist as cell surface proteins, anchored by their hydrophobic transmembrane domains, and can be processed to soluble forms by proteolytic digestion. Although all soluble forms of M-CSF were biologically active in murine bone marrow colony and proliferation assays, they showed greatly reduced or no activity in similar assays using human bone marrow.

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Transduction of the macrophage colony-stimulating factor gene into human multidrug resistant cancer cells: enhanced therapeutic efficacy of monoclonal anti-P-glycoprotein antibody in nude mice.

Sone S, Tsuruo T, Sato S, Yano S, Nishioka Y, Shinohara T.

Third Department of Internal Medicine, Tokushima University School of Medicine.

To develop a therapeutic modality for overcoming multidrug-resistant (MDR) cancer with anti-MDR1 antibody, we examined the effect of macrophage colony-stimulating factor (M-CSF) gene transfection into MDR AD10 cells on therapy of MDR cancer with anti-MDR1 antibody (MRK17) in nude mice. MDR human ovarian cancer (AD10) cells were transduced with the human M-CSF gene inserted into an expression vector to establish gene-modified cells capable of producing low (ML-AD10), intermediate (MM-AD10) nd high (MH-AD10) amounts of M-CSF. Systemic administration of MRK17 resulted in significant dose-dependent inhibition of subcutaneous growth of ML-AD10 tumors. In contrast, systemic administration of recombinant M-CSF in combination with MRK17 did not augment the therapeutic efficacy of MRK17 alone, but rather promoted the growth of the parent AD10 cells. To test the efficacy of in vivo M-CSF gene therapy combined with antibody, we mixed the parent AD10 cells with MH-AD10 cells producing a large amount of M-CSF, and inoculated the mixed cells subcutaneously. Treatment with MRK17 inhibited growth of the mixed cells more than that of the parent cells alone. Thus, combined therapy with anti-MDR1 mAb and M-CSF gene modification of MDR cancer cells may provide a new immunotherapeutic modality for overcoming MDR in humans.

PMID: 8698627 [PubMed - indexed for MEDLINE]

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Membrane interleukin 1 induction on human endothelial cells and dermal fibroblasts.

Kurt-Jones EA, Fiers W, Pober JS.

Department of Pathology, Brigham and Women's Hospital, Boston, MA 02115.

Human endothelial cells and dermal fibroblasts both expressed a membrane-associated interleukin 1 (IL-1) activity when stimulated with either recombinant tumor necrosis factor (TNF) or recombinant lymphotoxin but stimulated endothelial cells expressed significantly more membrane IL-1 per cell than did fibroblasts. Lipopolysaccharide induced membrane IL-1 activity on endothelial cells but not fibroblasts. Interferon-gamma treatment of endothelial cells and fibroblasts had no direct effect on membrane IL-1 expression and little effect when used as a pretreatment for TNF or lipopolysaccharide stimulation. Endothelial cell membrane IL-1 activity was induced within 24 hr of culture with TNF or lipopolysaccharide, and increased up to 72 hr of incubation. Antibodies raised against human monocyte-derived IL-1 species neutralized the membrane IL-1 activity of TNF-stimulated endothelial cells. Both absorption studies and neutralization with specific sera indicated that endothelial cell membrane IL-1 is structurally related to IL-1 alpha. Endothelial cells expressed both IL-1 beta mRNA in response to TNF, lymphotoxin, and recombinant IL-1 species, as detected by Northern blot analysis. These studies demonstrate that endothelial cells can be activated to express a cell-surface IL-1 activity which is structurally, as well as functionally, related to the secreted form of IL-1.

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Human recombinant macrophage colony stimulating factor activates murine Kupffer cells to a cytotoxic state.

Curley SA, Roh MS, Kleinerman E, Klostergaard J.

Department of General Surgery, University of Texas M.D. Anderson Cancer Center, Houston.

Activated macrophages mediate cytotoxicity against tumor targets and thus may modulate development and growth of metastatic tumor cells. Macrophage colony stimulating factor (M-CSF) has a potential role in activating mature macrophages to a cytotoxic state. We employed a murine Kupffer cell (KC) model of cytotoxicity against a tumor necrosis factor (TNF) - sensitive murine colon adenocarcinoma cell line (MCA26) to evaluate the ability of recombinant human M-CSF (rhM-CSF) 1) to act alone as a KC-activating agent and 2) to enhance KC cytotoxicity against MCA26 cells in association with known macrophage activating compounds. rhM-CSF produced a dose-dependent increase in TNF release by KC in vitro with a parallel increase in MCA26 killing. KC activated by rhM-CSF produced less TNF and concomitantly demonstrated a lower cytotoxicity against MCA26 cells when compared with KC activated by gamma interferon (gamma IFN) with or without lipopolysaccharide (LPS). M-CSF did not act in a synergistic fashion with gamma IFN and LPS to increase TNF secretion or cytotoxicity against MCA26 cells. rhM-CSF thus acts as a single agent capable of activating murine KC to a cytotoxic state but does not cooperate with classical priming/triggering signals to achieve KC activation.

PMID: 2118971 [PubMed - indexed for MEDLINE]

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M-CSF gene transduction in multidrug-resistant human cancer cells to enhance anti-P-glycoprotein antibody-dependent macrophage-mediated cytotoxicity.

Heike Y, Sone S, Yano S, Seimiya H, Tsuruo T, Ogura T.

Third Department of Internal Medicine, University of Tokushima School of Medicine, Japan.

A human macrophage-colony-stimulating-factor (M-CSF) gene inserted into an expression vector (pRc/CMV-MCSF) was transfected into multidrug-resistant (MDR) human ovarian cancer cells (AD10) to induce secretion of human M-CSF into the medium. The M-CSF level in the culture medium of the transfected cells reached 100 ng/ml after 7 days, and the ability of the cells to secrete M-CSF was stable for at least 3 months. Transfection of the M-CSF gene did not result in any change in expression of MDRI (P-glycoprotein), proliferation or chemosensitivity of the cells from those of the parent cells. There was also no difference between the transfected and the parent cells in susceptibility to NK cell- or interleukin-2-activated killer-cell-mediated cytotoxicity. Human blood monocytes that had been incubated for 4 days in medium with the culture supernatant of MH-AD10 cells exhibited higher ADCC activity than untreated monocytes against MDRI-positive cancer cells. This effect of the supernatant of AD10 cells was completely abolished by its treatment with a monoclonal anti-M-CSF antibody (MAb). When transfected human MDR cells were injected into nude mice, an inverse correlation was seen between the ability of the cells to produce M-CSF and their tumorigenicity. Thus, gene modification of MDR cancer cells seems hopeful as a therapeutic method for enhancing anti-MDRI-MAb-dependent macrophage-mediated cytotoxicity against human MDR cancer cells.

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Macrophage colony-stimulating factor complementary DNA: a candidate for gene therapy in metastatic melanoma.

Walsh P, Dorner A, Duke RC, Su LJ, Glode LM.

(Department of Dermatology), University of Colorado Cancer Center, University of Colorado Health Sciences Center, Denver 80262, USA.

BACKGROUND: At present, there is no highly effective treatment for metastatic melanoma. Innovative approaches aimed at inducing a more effective immune response against tumors have shown promising results in animal models. One approach involves the genetic modification of tumor cells so that they produce cytokines that stimulate an immune response. PURPOSE: The aim of this study was to determine the effectiveness of cytokine gene therapy for metastatic melanoma in a murine melanoma model. METHODS: B16F10 murine melanoma cells, which readily metastasize to the lungs, were transduced with a retroviral vector containing genes encoding neomycin resistance and human macrophage colony-stimulating factor (M-CSF). The presence of M-CSF messenger RNA in transduced cells was examined by coupled reverse transcription and polymerase chain reaction. Concentrations of soluble M-CSF in cell culture supernatants were determined by enzyme-linked immunosorbent assays (ELISAs). A clonal cell line, designated N+/CSF+, that expressed and secreted M-CSF was identified. Another clonal cell line, designated N+/CSF-, did not secrete M-CSF at levels detectable by ELISA. B16F10, N+/CSF-, and N+/CSF+ cells, individually or in combination, were injected intravenously or subcutaneously into C57BL/6 mice; we then evaluated the tumorigenicity and metastatic behavior of the cells, as well as the immune responses and survival of the mice. The immune responses assayed were the cytotoxic T lymphocyte (CTL) and peritoneal exudate cell (PEC) tumoricidal activities. RESULTS: Injection of B16F10 cells into the tail vein of C57BL/6 mice led to the establishment of lung metastases by week 2 and death by week 8. Injection of the N+/CSF+ or N+/CSF- cells led to the establishment of lung metastases that were detected at 2 and 3 weeks, respectively; however, these metastatic lesions were eliminated, and the

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animals had survival rates similar to those of the noninjected control mice. Injection of mice with a mixture of B16F10 and N+/CSF- cells resulted in the development of metastatic disease and 0% survival at 8 weeks, whereas mice that had been given an injection of a mixture of B16F10 and N+/CSF+ cells had an 80% survival rate at 8 weeks and survived at least two times longer (P = .007). The CTL and PEC tumoricidal activities in animals given an injection of N+/CSF+ cells suggested that monocytes and lymphocytes were responsible for the observed antitumor response. CONCLUSION: These findings suggest that the expression of M-CSF by genetically modified melanoma cells caused an effective antitumor immune response in host C57BL/6 mice and, thus, prolonged survival over that observed in the control mice.

PMID: 7791230 [PubMed - indexed for MEDLINE]

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Macrophage colony-stimulating factor gene transfer into tumor cells induces macrophage infiltration but not tumor suppression.

Dorsch M, Hock H, Kunzendorf U, Diamantstein T, Blankenstein T.

Institut fur Immunologie, Universitatsklinikum Steglitz, Freie Universitat Berlin, FRG.

In order to analyze the effect of a high local concentration of macrophage colony-stimulating factor (M-CSF; CSF-1) on tumor growth, the plasmacytoma cell line J558L was transfected with the human M-CSF gene and injected into syngeneic BALB/c mice. In contrast to the parental tumors, M-CSF transfectants were heavily infiltrated by macrophages as evidenced by immunohistochemistry with antibodies to Mac-1 and Mac-3 and by isolation of the macrophages from the tumor. Nevertheless, tumor growth was only slightly affected by M-CSF and M-CSF-producing cells grew as tumor in all cases. The growth retardation of M-CSF-producing cells varied depending on the experiment and seemed to be due to an indirect effect because the growth rate of the cells in vitro had not changed upon gene transfer. Attempts to activate the tumor-infiltrating macrophages for tumor suppression by systemic application of interferon-gamma and/or lipopolysaccharide were not successful. Altogether, our results suggest that M-CSF is a potent chemoattractant for macrophages in vivo but alone is not sufficient to activate these macrophages for tumoricidal activity.

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